When, where and how? Focus on neuronal calcium dysfunctions in Alzheimer's Disease.

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When, where and how? Focus on neuronal calcium dysfunctions in Alzheimer's Disease

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ABSTRACT

Alzheimer's disease (AD), since its characterization as a precise form of dementia with its own pathological hallmarks, has captured scientists' attention because of its complexity. The last 30 years have been filled with discoveries regarding the elusive aetiology of this disease and, thanks to advances in molecular biology and live imaging techniques, we now know that an important role is played by calcium (Ca^{2+}). Ca^{2+}, as ubiquitous second messenger, regulates a vast variety of cellular processes, from neuronal excitation and communication, to muscle fibre contraction and hormone secretion, with its action spanning a temporal scale that goes from microseconds to hours. It is therefore very challenging to conceive a single hypothesis that can integrate the numerous findings on this issue with those coming from the classical fields of AD research such as amyloid-beta (Aβ) and tau pathology. In this contribution, we will focus our attention on the Ca^{2+} hypothesis of AD, dissecting it, as much as possible, in its subcellular localization, where the Ca^{2+} signal meets its specificity. We will also follow the temporal evolution of the Ca^{2+} hypothesis, providing some of the most updated discoveries. Whenever possible, we will link the findings regarding Ca^{2+} dysfunction to the other players involved in AD pathogenesis, hoping to provide a crossover body of evidence, useful to amplify the knowledge that will lead towards the discovery of an effective therapy.

Keywords:

Alzheimer's disease, calcium homeostasis, amyloid-beta, VOCCs, nAChRs, glutamate receptors, CALHM1, SOCCs, endoplasmic reticulum, mitochondria, lysosomes.

INTRODUCTION

When the Ca^{2+} hypothesis of AD arose during the late '80s and early '90s, it was somehow disconnected from the amyloid cascade hypothesis that was already moving its first steps [1] [2]. When, later in the decade, studies linking Aβ accumulation and deposition to Ca^{2+} dysregulation begun to appear the amyloid and the Ca^{2+} hypotheses became linked [3]. It was soon clear that not only amyloid plaques but also soluble Aβ aggregates, and specifically Aβ oligomers (Aβo), were exerting significant toxicity, either endogenously produced or exogenously administered to cells and primary neurons in culture [4][5] [6] (**Figure 1**). The Ca^{2+} hypothesis of AD, by positing intracellular Ca^{2+} dysregulation as the direct consequence of Aβ toxicity, offered one of the richest grounds for researchers in the field. It obscured the fact, however, that Ca^{2+} dysregulation at the onset of AD can occur independently of Aβ accumulation and toxicity [7]. AD is now considered as a multiple hits disease where amyloid and tau dysfunctions are aggravated by oxidative stress, intracellular Ca^{2+} imbalance and metabolic disturbance. A large body of evidence strongly points towards Ca^{2+} as a possible unifying element underlying AD pathophysiology. According to the amyloid cascade hypothesis, Aβ toxicity precedes tau pathology but exactly how Aβ and tau cooperate in synaptic dysfunction is still a controversial issue [8]. Studies with AD patients as well as with amyloid precursor protein (APP) and/or tau mouse models have shown that tau defects affect the lateral entorhinal cortex at an early stage but require Aβ to reach other cortical sites [9].

In this contribution, we will mainly focus on the relationship between cellular Ca^{2+} handling and AD, paying special attention to the crosstalk with Aβ. Given the large amount of data collected, the often contrasting hypotheses and the vast array of cellular players more directly involved in Ca^{2+} homeostasis, the review will be divided in thematic paragraphs, based on the "anatomy" of the Ca^{2+} signal (**Figure 2**).

2. PLASMA MEMBRANE CHANNELS

It was initially suggested that Aβ peptides are capable of forming plasma membrane channels [10]; indeed, remarkable increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) were reported upon challenging cultured cells with either Aβ40 or Aβ42 [11] [12] [13]. The "ion channel hypothesis" for Aβ toxicity, as proposed by Arispe's group, was mainly supported by *in vitro* studies demonstrating the formation of cation selective channels, whose permeation is inhibited by metals such as Zn^{2+} and Cu^{2+} and by particular peptides designed to line the pore-forming region [14] [15]. The ion channel hypothesis got further support from Demuro's studies showing that, in oocytes, Aβ injection causes cytosolic Ca^{2+} rises due to Ca^{2+} entry across Aβ channels formed in the plasma membrane as well as Ca^{2+} release via stimulation of the Gq/PLC pathway [16]. This hypothesis contrasts with *in vivo* data showing that exogenously applied Aβ enhance the overall plasma membrane permeability to anions and cations rather than forming a Ca^{2+}-selective pathway [17]. Since the first observations describing the increase in the $[Ca^{2+}]_{cyt}$ of cells directly harvested from AD patients [18], many groups had focused their attention on this phenomenon changing the paradigm from the channel-forming to the channel-modulating hypothesis. This intense effort led to a plethora of hypothetical candidates capable of mediating cellular Ca^{2+} overload by Aβ since neuronal cells have a vast array of Ca^{2+}-permeable plasma

membrane channels at their disposal. As suggested players implicated in the pathogenesis of AD, and possibly mediating Aβ toxicity, we will here consider the voltage-operated Ca^{2+} channels (VOCCs), the nicotinic acetylcholine receptors (nAChRs), the ionotropic glutamate receptors [N-Methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs)], the Ca^{2+} homeostasis modulator 1 (CALHM1), as well as the store-operated Ca^{2+} channels (SOCCs). For a thorough investigation on this issue, also including the plethora of Aβ binding receptors, the readers should refer to excellent more extensive reviews of which only a few are cited here.

2.1. VOCCs

VOCCs open following membrane depolarization: the firing of an action potential by the neuron, that is the integrated answer of the afferent presynaptic stimuli, causes the plasma membrane to depolarize, allowing Ca^{2+} to enter from the outside along its huge electrochemical gradient. This Ca^{2+} signal causes the opening of a subtype of potassium channels that helps membrane repolarization. Ca^{2+} entry is coupled with synaptic vesicle release and intracellular signalling cascades that lead to gene transcription of several players involved in synapses' maintenance and plasticity.

VOCCs, especially those of the L-type subfamily, have long been implicated in aging and AD. Several studies have reported an elevated activity of these channels, with a subsequently larger Ca^{2+}-dependent hyperpolarization, as a possible explanation for the deterioration of synaptic efficiency [19][20] [21] [22]. Given that the Ca^{2+} signal is spatially and temporally well defined, it is conceivable that even a little delay in its clearance from the cytosol after depolarization can have dramatic effects; for instance, the differential activation of the Ca^{2+}-Calmodulin-dependent kinase II (CaMKII) and the phosphatase calcineurin (CN) is based on their different reaction times to cytosolic Ca^{2+} elevations [23] [24].

An impaired intracellular Ca^{2+} handling, that derives either from an increased and sustained entry through the membrane channels or from an inefficient clearance, can lead to an imbalance between long-term potentiation (LTP) and long-term depression (LTD), the electrophysiological correlates that support learning and memory, thus eventually resulting in disbanded memory storage. L-type ion channels are required for LTP at synapses between mossy fibres and CA3 pyramidal neurons. This type of LTP does not depend on NMDARs and involves postsynaptic Ca^{2+} influx carried by L-type VOCCs [25]. Patients with AD show higher L-type VOCC expression in the hippocampus compared with healthy individuals [26]. Forette and co-workers found that the blockage of L-type VOCCs with dihydropiridines, a class of drugs mainly used to treat systolic hypertension, was able to prevent dementia in some AD patients [27]. Consistently, in CA1 neurons of the hippocampus, Ca^{2+} currents through L-type VOCCs increase during aging in rats, together with a marked decline in learning and memory faculties. Chronic nimodipine treatment ameliorates memory loss, suggesting that excessive Ca^{2+} influx, through L-type Ca^{2+} channels, impairs learning and memory [28]. However, it has been demonstrated that in cultured neurons from $APP_{sw,Lon}$ mice, Aβ suppress gene transcription that is mediated by Ca^{2+} influx (and cAMP surge) and agonists of L-type VOCCs efficiently reverse these transcriptional deficits, implicating a defect in Ca^{2+} influx rather than an augmentation [29]. Furthermore, in cell lines and primary cerebellar

granules from the Tg2576 AD mice, dihydropyridines, and especially the most used nimodipine, increase Aβ42 secretion through a mechanism that is independent of Ca^{2+} entry-blockage thus raising doubts to the possible therapeutic use of these compounds [30]. The effect of Aβ on L-type VOCCs, whenever present, is likely dose- and time-dependent, since a 1-hour exposure of mouse cortical wilt-type neurons to Aβ42o at submicromolar concentrations does not affect Ca^{2+} peaks and long lasting plateaus due to membrane depolarization [31]. Interestingly, Aβ globulomers (the 48-kDa component of Aβo) suppress spontaneous synaptic activity of both GABAergic and glutamatergic synapses by acting selectively on presynaptic P/Q type Ca^{2+} currents [32]. At the presynaptic level, Aβo also affect Ca^{2+} influx through VOCCs, possibly explaining fast axonal transport defects [33].

2.2. nAChRs

Aβ were reported to affect cholinergic neurons precociously. In the central nervous system, their cell bodies are packed in dense nuclei in the basal forebrain whilst their axonal projections spread throughout the whole cortex, exerting a modulatory effect rather than pure excitation. Acetylcholine binds to two families of receptors, the ionotropic nicotinic receptor (nAChR) and the metabotropic muscarinic one (mAChR). The first one is a Ca^{2+}-permeable non-selective cation channel with a pentameric structure being formed by alpha or beta subunits, or a mix of the two. The subunit composition influences the pharmacological and kinetic properties of the channel, varying its permeability to K^+, Na^+ and Ca^{2+} [34].

Ionotropic nAChR exist in various forms, among which the homomeric (α7) and the heteromeric α4β2-nAChR were the first to be investigated in AD and they still represent the most promising targets [35] [36]. Recently an heteromeric α7β2 nAChR has been added to the list of Aβ-binding nAChRs [37]. They differ in their Ca^{2+} permeability and sensitivity to α–bungarotoxin, and, functionally speaking, they are thought to take part in memory formation and attention and they have been long known to interact with Aβ. In particular, several studies demonstrated the different affinity and exerted effects upon binding to Aβ40 or Aβ42 by the α7-nAChR and the α4β2-nAChR [38] . Summarizing, we can say that both receptor subtypes bind Aβ, preferentially the 42 amino-acid long peptides, which activate α7-nAChR in the picomolar range and inhibit it in the nanomolar one, whilst α4β2-nAChR binds Aβ only in the nanomolar range with an inhibitory effect. Given that amyloid deposition is a spatially defined phenomenon, with certain brain areas exposed to premature deposition, it is conceivable that widely different interstitial Aβ concentrations can be achieved, causing diverse effects on nAChRs. Albeit contrasting with regard of the Aβ concentrations, species (oligomers *vs* monomers) and models used, almost all the evidence points towards a disruption of the physiologically nAChRs-driven LTP, which predicts an impairment in memory formation [39]. This was tested further by generating *in vivo* models bearing AD-related APP mutations in a α7-nAChR knock-out (KO) context. However, depending on the APP mutations employed, different results were obtained. The KO of α7-nAChR in a $APP_{swe,Indiana}$ mouse model was protected against synaptic loss and memory deficits, when tested at 13 and 16 months of age [40], while the same KO in a APP_{swe} mouse model worsened both memory and Aβ pathology, with increased amount of

soluble high molecular weight (HMW) oligomers when tested at 5 months of age [41]. To our knowledge, no studies were conducted to shed light on the intracellular pathway involved in these effects and, therefore, Ca^{2+} contribution is inferred only on the basis of the high Ca^{2+} permeability of neuronal nAChRs. Indeed, a likely recruitment by Aβ of astrocytic α7-nAChR, altering both Ca^{2+} homeostasis and gliotrasmission, further complicates the picture [42] [43].

2.3. Ionotropic Glutamate Receptors

A vast majority of excitatory neurons rely on glutamate as neurotransmitter and are therefore defined glutamatergic. Glutamate exerts its excitatory effect interacting with a diverse array of ionotropic receptors (NMDARs, AMPARs, KainateRs) and metabotropic receptors (mGluRs). In the AD/aging field, the best characterized is the NMDAR since it is pivotal in LTP. It has been proposed that the interaction of Aβo with NMDARs is an initiating/propagating event in the pathogenesis of AD. A high percentage of the reports concerning the physical and functional interactions between Aβo and NMDARs relies on synthetically prepared, poorly characterized oligomers, thus leading to contrasting and misleading results. Co-immunoprecipitation with the NMDAR subunit NR1, together with genetic knockdown of the latter and pharmacological blockade of the whole channel had proven that Aβo preferentially target the synapses due to the presence of NMDARs [44] [45]. Recently, it was shown that soluble Aβ assemblies derived from the brains of individuals with AD interact with cellular prion protein (PrP^C) to activate Fyn [46] [47]. Aβ engagement of PrP^C-Fyn signalling requires mGluR5 as co-receptor, which leads to phosphorylation of the GluN2B subunit, culminating in loss of surface NMDARs and dendritic spines [48]. Furthermore, PrP^C KO prevents the toxic effects on LTP ascribed to Aβo [48]. Nonetheless it was reported that PrP^C exerts a modulatory effect on the glutamatergic signalling, also in physiological conditions, with neurons from a PrP^C null mice exhibiting sustained and aberrant NMDAR mediated currents [49]; a competition between PrP^C and Aβo for the modulation of the NMDAR activity could be present since they exert opposite effects on glycine binding at the co-agonist site, with PrP^C negatively modulating it [50].

It has recently been suggested that Aβo, by triggering astrocytic release of glutamate to the extrasynaptic space, preferentially affect extra-synaptic GluN2B-NMDARs that, at variance with synaptic GluN2A-NMDARs, further promote the amyloidogenic pathway and Aβ production [51]. The increased amyloid burden can eventually lead to neuronal toxicity by inhibition of excitatory amino acid transporters (EAATs). The subsequent increase in the extracellular concentration of glutamate in turn activates its receptors in an aberrant way; albeit never demonstrated, the interaction between Aβo and EAATs is extremely likely and there are studies reporting an altered expression pattern of EAATs in the AD brain [52] . Taken together, these findings suggest a neurotoxic action mediated by Aβo through the disbandment of the excitatory glutamatergic transmission. However, the only strong evidence at the *in vivo* level is the positive effect of the weak NMDAR antagonist memantine, which has long been used in AD treatment, and its successor nitro-memantine. Both are efficient at blocking or reversing the deleterious actions of Aβo largely because of their selectivity for extrasynaptic NMDARs [53] [51]. The hypothesis of Aβ toxicity involving extra-synaptic NMDARs got only

partial validation given that the GluN2B-NMDAR antagonist Ro 25-6981 is protective against excitotoxic neuronal death and Aβ-induced synapse loss in some AD mouse models [54][55], and was not confirmed in others [56].

Among other intracellular events prompted by Aβo, the activation of calcineurin and the dephosphorylation of NMDARs and AMPARs are primary mechanisms prompting receptor internalization [57][58][59]. Of note, the synaptic loss induced by Aβo through NMDAR activation seems independent of Ca^{2+} influx, relying mainly on p38-MAPK activation [60].

An issue of extreme importance, that has unfortunately gone almost unseen, is the fact that a substantial amount of data on the interaction between Aβo and glutamate receptors come from studies employing synthetic Aβo that have been prepared by dissolving and aging the monomers in DMEM/F12 medium [61]. The latter type of medium specifically causes cytosolic Ca^{2+} increases on its own, likely because of its high content in glutamate and, especially of glutamine that undergoes degradation during the aging procedure. It has been estimated that a 100 μM stock solution of Aβo aged in DMEM-F12 medium might contain up to 100 μM glutamate [62]. Using Aβo at concentrations equal or above 1 μM thus means stimulating cells with glutamate at concentrations sufficient to elicit Ca^{2+} rises through NMDA- and mGlu-Rs [62]. With this caveat, data interpretation is extremely complicated. Moreover, Aβo were often administered to neuronal cultures or slices, at concentrations considerably higher than those found in the AD brain (5-10 μM). Neuronal Ca^{2+} rises, due to addition of different culture media used for preparing Aβo, were recently described also by Caballero et al. [63]. The same authors show that culture media devoid of glutamate and glutamine but containing sub-micromolar amounts of Zn^{2+}, Cu^{2+}, Fe^{2+} allow production of low molecular weight (LMW) oligomers that are responsible for cytosolic and mitochondria Ca^{2+} overload [63]. We have recently demonstrated that neither synthetic Aβ monomers nor oligomers (ranging from low to high MW), when aged in distilled water, can elicit neuronal Ca^{2+} increases when acutely applied to neuronal cultures and PC12 cells at concentrations ranging from 0.5 to 5 μM (**Figs 3,4**) [31]. Conversely, a 1-hour exposure of cortical neurons to Aβo profoundly disturbs neuronal Ca^{2+} stores through a pathway that involves mGluR5 but not NMDARs [31]. Our findings point to a more subtle but insidious effect of Aβo, rather than to a massive Ca^{2+} load. Indeed, the idea that a long-lasting disease, such as AD, might act simply through the disruption of a neurotransmission as ubiquitous as the glutamatergic one, shed shadows on a generalized, massive involvement of neuronal glutamate receptors, and forces to investigate AD dysfunctions also at different levels such as the glia and the neurovascular unit [64][65].

2.4. CALHM1

Another relevant player in the crosstalk between Ca^{2+} and Aβ is the Ca^{2+} homeostasis modulator 1 (CALHM1), whose polymorphism P86L was shown to segregate with early-onset forms of sporadic AD [66]. Initially thought to be a Ca^{2+}-selective entry pathway and later proposed as an ATP-exiting pore majorly involved in taste transmission [67], CALHM1 is also more generally implicated in neuronal excitability [68]. Interestingly, the channel shares homology with connexins, pannexins and innexins [69]. It was also described as a relevant

component of neuronal ER Ca^{2+} leak, which is blocked by a class of compounds similar to benzodiazepines [70][71]. In *Calhm1* knockout mice, Aβ accumulation is increased by 50% because of reduced clearance by insulin degrading enzyme; evidence was provided showing that channel activation and Ca^{2+} influx are required for the antiamyloidogenic effect of CALHM1 [72][73].

2.5. SOCCs

All the major components (Orai and STIM proteins) of the Store-Operated Ca^{2+} Channels (SOCCs) have been described at the neuronal level and a significant amount of studies have underlined the relevance of Capacitative Ca^{2+} Entry (CCE) on the pathophysiology of the CNS [74][75][76] and AD [77][78]. Notwithstanding, very little is known on the effects of Aβo at this level. As originally reported by Verkhrasky and coll. [79], in central but not peripheral rat neurons, caffeine-sensitive Ca^{2+} stores are almost empty at rest, likely because of a highly dynamic ER Ca^{2+} efflux, caused by the ongoing neuronal spiking activity and neurotransmitter release. Despite the fact that these neurons can efficiently refill their store Ca^{2+} content during KCl-evoked depolarization, they lose the releasable Ca^{2+} very quickly [79]. Similarly, in mouse cortical neurons IP_3-sensitive Ca^{2+} stores are very labile, and can be maximally refilled by prolonged depolarization [80]. On these premises it is expected that CCE, to name all the Ca^{2+} entry pathways linked to store depletion, is constitutively active at rest [81]. We have recently shown that acute treatment of mouse cortical neurons with Aβ before the refilling process does not significantly modify the store Ca^{2+} content, suggesting that neuronal CCE is not acutely blocked by soluble Aβo [31]. We can also exclude that a 1-hour exposure to Aβo exerts an indirect effect on CCE given that the total store Ca^{2+} content is not affected, as assayed by ionomycin treatment [31]. Taken together, we do not envisage a direct effect of Aβo on CCE. Indeed, the opposite is likely true, with CCE having a direct effect on Aβ secretion: a high Ca^{2+} influx through CCE reduces Aβ secretion, while a low influx increases it [82]. Given the fact that FAD-linked mutant PSs reduce CCE, their effect on Aβ accumulation is twofold: favouring Aβ production as well as secretion [83]. Recently, it has been shown that the STIM2/neuronal SOC/CaMKII pathway is down-regulated in PS1KI mice, a condition that leads to loss of mushroom spines, the "stable memory spines" that are directly involved in long-term memory by making functionally stronger synapses [84]. The same group similarly demonstrated that, in hippocampal neurons of APPKI mice, mushroom spine loss results as accumulation of extracellular Aβ42 [85]. The effect is due to overstimulation of mGluR5 by Aβ42, causing ER Ca^{2+} overload and consequently STIM2 down-regulation [85]. Unfortunately, the Aβo employed in that study were aged in F12 medium raising doubts about the effective toxic agent. Of note, the ER Ca^{2+} overload induced by Aβo, described in APPKI neurons [85], is a chronic and indirect effect, since different FAD-linked APP mutations modify neither the cytosolic nor the store Ca^{2+} level ([86]) but see also [87] for a reduced ER Ca^{2+} loading capacity in APP_{swe}-expressing cells.

It is worth noting that a major role for CCE is expected in astrocytes and microglia cells, whose signalling is largely based on ER stores. Interestingly, a reduction in Ca^{2+} release and sustained Ca^{2+} influx upon agonist stimulation was reported in microglia of AD patients compared to non-demented individuals [88] whereas chronic incubation of astrocytes with Aβo at nanomolar concentrations potentiates Ca^{2+} influx caused by

activation of mGluRs [89].

By using plasma membrane-targeted aequorin, it was recently shown that in mouse cerebellar granules, PrP^C exerts a tonic inhibition on SOC Entry channels by reducing the level of STIM1 phosphorylation [90]. It was suggested that Aβo target mGluRs through the interaction with PrP^C [91][48] one of the major Aβo binding sites. Of interest, potentiation of SOCE by Aβo is lost in PrP^C-KO mice [90].

3. ENDOPLASMIC RETICULUM CHANNELS

Amidst the complex scenario of ER Ca^{2+} dysregulation, another set of findings has shaken the scientific community over the past 15 years. At the beginning of the 21st century, several groups reported that presenilins (PS1 and PS2), the catalytic subunits of the γ-secretase, are able to modify the ER Ca^{2+} handling when overexpressed in cell lines (see for reviews [83][92]). Among the most successful sets of studies was that produced by Bezprozvanny and co-workers, who defined the well-known paradigm of the "Ca^{2+} overload" hypothesis of AD [93]. According to this model, endogenous wild-type (wt) PSs work as the elusive ER Ca^{2+} leak channel that has puzzled scientists for decades, a function that is impaired when one PS allele bears a FAD-linked mutation. This situation leads to accumulation of Ca^{2+} inside the ER and consequently exaggerated, toxic release when the IP3Rs and RyRs open. This fascinating hypothesis has been questioned both by ourselves and other groups [94]. In fact, by using organelle-targeted Ca^{2+} probes, we first discovered that different types of PS2 mutants reduce the ER and Golgi apparatus Ca^{2+} content, with PS1 mutants exerting no effect or even partially mimicking the PS2 mutants [80][95] [7][96]. These latter exert their effects through a reduction of the SERCA pump with minor effects on ER Ca^{2+} leak [97]. By using both cytosolic and ER Ca^{2+} probes, we provided evidence that not only cell lines but also neurons in cultures and slices from PS2-N141I transgenic mice display a reduced store Ca^{2+} content and responsiveness to IP3-generating agonists, albeit in the presence of larger responses to ryanodine receptor (RyR) activation by caffeine [98] [99]. No clear differences in ER Ca^{2+} levels and dynamics were found between wt and PS double knockout (DKO) MEFs [97]. Furthermore, Foskett and coworkers similarly found no clear evidence of ER Ca^{2+} overload due to the expression of PS1 or PS2 mutants [94], and partly solved the issue demonstrating that the "apparent" Ca^{2+} overload is due to exaggerated Ca^{2+} release as a consequence of IP3R-gating modulation by PS mutants [100]. Moreover, they demonstrated that in 3xTg mice, reduction of IP3R1 expression levels attenuates Aβ accumulation and tau hyper-phosphorylation and rescues hippocampal LTP and memory deficits [101]. In spite of the conflicting findings related to ER Ca^{2+} content and IP3R release, it might be possible to envisage a unifying hypothesis that sees the RyR over-expression/activation as a common pathway that brings together PS1 and PS2 mutants as well as Aβo [102][103]. Notably, dantrolene, a RyR1 blocker used to treat malignant hyperthermia, has been reported to soften the symptoms and to reduce the amyloid burden in AD mouse models [87][104]. Since RyR is present in three different isoforms, all of them represented at the brain level, and due to the lack of commercially available specific antibodies, it is difficult to prove which specific isoform is involved. In the previous work, using quantitative PCR (qPCR), the potentiated RyR response was attributed to upregulation of RyR2, the isoform also linked to synaptic loss and neuronal death [105][106]. Increased

levels of RyR2 also characterize the mouse brain of transgenic mice based on mutant PS2 [99]. In contrast, the RyR3 isoform, which exerts a pro-survival effect, was not involved [107].

4. THE MITOCHONDRIA CHANNELS

Mitochondria are currently considered among the primary targets and players in neurodegenerative diseases, including AD, with mitochondrial dysfunctions being considered a hallmark of Aβ-induced neuronal toxicity. It has been shown that soluble Aβ accumulate precociously in neuronal mitochondria of Tg2576 mice [108]. Additionally, APP has been suggested to enter the mitochondrial protein import machinery (TOM/TIM) with its C-terminal domain, leaving the huge N-terminal domain outside [109]. The APP intracellular domain (AICD) is then cleaved by mitochondrial γ-secretases and dysfunctions might thus arise from P3 and Aβo generated inside the mitochondrial matrix [110]. *In vitro* studies have shown that incubation of Aβo with cells or purified mitochondria leads to decreased mitochondrial function [111]. Others have described deleterious effects of Aβo on the outer mitochondrial membrane (OMM) where, by activating the apoptotic BAK pore, they induce cytochrome *c* release and cell death [112]. Aβo interaction with BAK on the OMM is difficult to conceive giving that Aβo do not display a cytosolic localization, being found extracellularly, following both production at the plasma membrane (less than 10%) and active release, and intracellularly inside vesicles - endosomes and multivesicular bodies - where they are produced upon APP internalization and from which they cannot easily escape. Thus, under pathophysiological conditions, direct effects of Aβo on intracellular channels (MCU, PTP, VDAC, RyR, IP3R, ER leak channels, to mention a few) are not expected, unless they are translocated across membranes. Whereas Aβ mitochondrial localization and uptake were confirmed in brain human samples and in *in vitro* studies [108][109], mitochondrial localization of both APP and γ-secretase components is still waiting, leaving the topology conundrum of Aβ transfer across membranes yet an open question. Furthermore, it remains to be explained what physiological role APP is playing at the mitochondrial level.

Among the dysfunctions through which mitochondria are likely responsible for neuronal demise in AD are those occurring at the ER-mitochondria network level. We discovered that upon agonist stimulation, mitochondria from cell lines and neurons carrying FAD-linked human PS2 mutations experience much higher Ca^{2+} transients despite a lower ER Ca^{2+} content. The phenomenon is due to an increased number of ER-mitochondria close contact sites occurring at the level of mitochondria-associated ER membranes (MAMs), a finding reported also with PS2 but not PS1 mutants [99][113] [98][114][115]. Interestingly, Aβ can be produced at the MAM level and knocking down mitofusin-2 increases ER-mitochondria contact sites [116] and inhibits Aβ production [117].

Indirect effects on the expression level of VDAC1 and IP3R3, the corresponding ER facing IP3Rs, have also been reported for Aβ at nanomolar concentrations [113]. Indeed, the effect is rapid since a 1-hour incubation with Aβo is sufficient to significantly increase the IP3R3 mRNA level in cultured mouse cortical neurons (**Figure 5**).

How an increased ER-mitochondria coupling will ultimately cause pathological alterations at different levels

such as energy metabolism, lipid synthesis, mitochondria remodelling and apoptotic signalling has yet to be fully investigated.

5. THE LYSOSOMAL CHANNELS

A growing body of evidence has linked failure of the autophagic pathway to multiple outcomes in neurodegenerative disease. In AD, lysosomal Ca^{2+} storage/release defects accelerate amyloidogenesis, neuritic dystrophy and apoptosis. It is well known that vesicular Ca^{2+} is required for efficient vesicle fusion including lysosomal vesicles [118][119]. Lysosomal Ca^{2+} defects have been reported in $PSEN^{-/-}$ cells and neurons, thus suggesting an additional route through which PS dysfunctions impair cellular proteostasis and autophagic fluxes. Nixon and coworkers have shown that PS1 deficiency causes a primary defect in V-ATPase that determines lysosomal alkalinization [120]. Furthermore, the same authors have suggested that anomalous alkalinization causes hyperactivation of the transient receptor potential mucolipin-1 (TRPML1) channels located in the lysosomal membrane, thus inducing both organelle Ca^{2+} depletion and cytosolic Ca^{2+} overload [121]. Whereas in PSEN-DKO MEFs and $PSEN1^{-/-}$ hippocampal neurons defects in lysosomal Ca^{2+} content and fusion were fully confirmed, defective V-ATPase and lysosomal alkalinization were not reported [122]. How lysosomal Ca^{2+} depletion contributes to AD neuronal dysfunction and death is yet to be fully elucidated. Unfortunately, obtaining reliable measurements of Ca^{2+} dynamics inside acidic vesicles is still an unsolved challenge. Regarding AD mouse models based on PS2-N141I, we did not observe any significant effects of mutant PS2 on acidic Ca^{2+} pools: the combined use of ionomycin to release neutral stores, followed by monensin to collapse the proton gradient of acidic vesicles and allow ionomycin to release their Ca^{2+} content, demonstrated that the acidic pools of PS2-N141I expressing neurons have the same Ca^{2+} content of neurons from control mice [99]. Notwithstanding, small pH differences in acidic pools cannot be detect by this indirect approach.

6. CONCLUDING REMARKS

The brain consists of a vast array of interconnected neuronal and astrocytic cells. As such, the aforementioned intracellular Ca^{2+} dysregulations, when spread across different cell types and brain areas, can have a prominent effect on overall brain performance. Recent technical advances in Ca^{2+} imaging at the *in vivo* level, together with a deeper knowledge about the role of the different cell populations, have enabled us to look at the brain as a whole, spotting the effects of the subtle intracellular defects at the network level [123]. We also reported an increased susceptibility to a pro-epileptogenic protocol employing picrotoxin in acute slices of two-week old AD transgenic mice based on mutant PS2 [99], therefore highlighting the possibility that hyper-excitability and hyper-synchronicity are early network deficits that precede amyloid deposition [124]. Other works, focusing their attention on the astrocytic network of the APPswe/PS1ΔE9 AD mice and imaging Ca^{2+} waves *in vivo*, have shown that at 4-5 months of age astrocytes display an elevated resting Ca^{2+} level independent of plaque proximity. Nonetheless, spontaneous intracellular Ca^{2+} waves were observed in astrocytes close to

plaques, while they were able to spread over a distance of at least 200 μm [125]. The same authors found that neurons in close vicinity of Aβ plaques are characterized by an increased activity driven by Ca^{2+} dyshomeostasis and affecting both structure and function that leads to a final disruption of the neuronal network [126]. In the frontal cortex of APP23xPS45 mice, hyperactive neurons were found in proximity of plaques but without a direct contact with them, suggesting a halo effect of soluble intermediates [127]. Indeed, the same authors demonstrated a massive increase in overall hippocampal activity and an altered ratio between hyperactive and hypoactive hippocampal neurons in young APP23xPS45 mice in the absence of plaques, thus concluding that neuronal hyperactivity is an early neuronal dysfunction of AD mouse models [128]. The noxious stimulus is likely represented by Aβo such as dimers, trimers and higher order oligomers, species that exist before plaque formation and to whom plaques, once formed, represent a reservoir. In fact, local application of soluble Aβo in the form of synthetic dimers mimicked hyperactivity of CA1 neurons in wild-type mice *in vivo* [128]. However, these works do not distinguish the role of excitatory pyramidal neurons and inhibitory interneurons, an issue of profound importance when it comes to specific circuitry functionality. It is worth noting that AD patients show a 5- to 10-fold increased probability of experiencing epileptic seizures, which correlate with the progression of the cognitive decline [129][130]. The degree of neuronal activity might influence the level of Aβ plaques deposition: it has recently been reported that cortical areas associated with elevated basal activity are more prone to develop amyloid deposits, and that the active unilateral vibrissae hyper-stimulation causes an increased plaque burden in the controlateral barrel cortex [131].

Increased neuronal hyper-excitability was observed in hippocampal slices not only from PS2APP mice but also from single mutant PS2 mice, *i.e.*, with an extremely low level of Aβ-load. Our data suggest that either a few pg/mg of wet tissue of Aβ42 are sufficient to eliciting neuronal hyperactivity or that the ER Ca^{2+} defects themselves, due to the mutant PS2, are the initiating cause of altered network activity [99]. Curiously ER Ca^{2+} defects also characterize human fibroblasts obtained from FAD PS2-T122R twin patients, one of the two in the total absence of cognitive decline [7]. Taken together, these findings indicate that Ca^{2+} dysregulation, particularly at the store level, occurs precociously, likely accelerating the disease progression and could be instrumental to find early biomarkers of the disease onset.

At the brain network level, the concerted activity of excitatory and inhibitory neurons originates signals oscillating in different frequency domains. The correct storage and retrieval of memories in the hippocampus strongly relies on this rhythmic activity, which is most prominently featured by the so-called theta (4-12 Hz) and gamma (25-100 Hz) oscillations. Theta and gamma rhythms can interact in a way that is known as cross-frequency coupling, where the phase of the theta oscillation influences the amplitude of gamma wave. This synchronicity mechanism underlies the formation of the working memory, a functionality that is severely impaired in the AD patient [132][133]. These aspects of brain activity can be measured using a variety of methods, even on human subjects, but, in spite of their relevance, very little work has been carried out on this topic. It has recently been reported that, prior to plaque deposition, APP23 mice display a deeply altered rhythmic pattern, with an overall increased burst activity, met by a decreased theta rhythm power, an increased gamma component and an impaired cross-frequency coupling [134]. Both the balance between the two rhythms

and their coupling were influenced by riluzole, that blocks voltage-gated sodium channels, and MK-801, a NMDA receptor blocker, thus highlighting the importance of the activity-evoked Ca^{2+} signal [134]. Taken together, these results suggest how intracellular events linked to Ca^{2+} dysregulation, which affect neurons as well as other cell types in the brain, can ultimately lead to a diffuse dyshomeostasis on different activities, causing structural and functional damages that eventually result in brain dysfunction and cognitive decline.

Disclosure statement

The authors declare no conflict of interest.

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FIGURE LEGENDS:

FIGURE 1. Aβ aggregation pathways. Aβ42 can aggregate following different pathways that lead to different, albeit coexisting, products [121]. The left side shows a trimer-based aggregation process resulting in soluble aggregates of Low and High Molecular Weight (Aβo) that are responsible for a vast array of cellular effects [122]. The right side shows the pathway leading to the formation of insoluble fibrils. Both pathway eventually can lead to plaques. Modified from [135].

FIGURE 2. Synaptic Aβo targets. Aβo impair the functionality of several targets localized at the synaptic level. We have here reviewed some of the major pathways involving Ca^{2+} dynamics, among which: metabotropic glutamate receptor (mGluR), store-operated Ca^{2+} channel (SOCC), cellular prion protein (PrP^{C}), N-methyl-D-aspartate ionotropic glutamate receptor (NMDAR), nicotinic acetylcholine receptor (α7-nAChR), voltage-operated Ca^{2+} channel (VOCC). Other targets less directly involved on Ca^{2+} homeostasis such as the neuroligin-1 (NL-1) adhesion molecule and the low affinity Nerve Growth Factor receptor (p75), have not been included but see refs [123]. Only receptors located at the postsynaptic site are here highlighted; however, also targets at the presynaptic site should be considered to fully explain Aβo toxicity. Modified from [136].

FIGURE 3. Preparation of synthetic Aβ(1-42) monomers and oligomers. Human synthetic dry powdered Aβ(1-42) (Anaspec, Fremont, CA, USA) is dissolved in 1,1,1,3,3,3-hexaflouro-2-isopropanol (HFIP) at room temperature (RT) for 1 hour to final concentration of 200 μM and stored as 10-20 μl aliquots at -80˚C until use. Aβ42o and Aβ42m are prepared according to the following procedure: aliquots are spun for 10 minutes (13,000 rpm, 5 min, $4^{o}C$) and then the HFIP is carefully removed by air fluxing with a pipette for several times and allowed to dry for about 10 minutes. The dried HFIP film is dissolved in DMSO (500 μM) and then diluted (50 μM) in double distilled water. When used immediately, the stock solution contained mainly monomers and low molecular weight (LMW) oligomers (6-17 kDa) whereas when left at RT for 48-72 hours it also contained high molecular weight (HMW) oligomers (50-100 kDa) that disappeared upon boiling [124] as shown from the Western blot on the right side. Since no quantification of HMW oligomers is carried out, the level of oligomers in the mixture is expressed as the equivalent concentration of monomers. Cells are routinely exposed to either Aβ42m or Aβ42o (0.5 μM) by adding 5 μL of the stock solution (50 μM, 10% DMSO) to 0.5 mL of the bathing medium. Before cell treatment, the Aβ42 stock solution was spun (13,000 rpm, 10 min) and immediately used. Preparations are tested by transmission electron microscopy (TEM). Images are acquired on a Tecnai 12 microscope (FEI, Hillsboro, Oregon, USA) operating at 100kV. The stock Aβ42 solution (50 μM) of either oligomers or monomers is diluted 5 times in bi-distilled water, and 20 μl samples are placed on Formvar/carbon-coated nickel grids and blotted off after 2 minutes. Samples are negatively stained with uranyl acetate and observed at 90,000X magnifications. Aβ42o particle diameter mainly ranges from 5 to 15 nm, consistent with a HMW oligomer population; Aβ42m and LMW aggregates are not revealed by TEM [125].

FIGURE 4. Characterization of $A\beta_o$ by Western blot. Oligomeric preparations are separated by gel electrophoresis using a tris-tricine 4-10-16% polyacrilamide gel. Aβ42o are diluted in loading sample buffer

(LSB; in mM: 50 Tris, 50 tricine, 2% SDS, 12% glycerol, 80 dithiothreitol, pH 8.25 at RT) at the final concentration of 20 ng/µl. Samples (200-400 ng) were loaded in LSB on a 4-10-16% (w/v) Tris-Tricine discontinous gel in Tris-Tricine buffer with or without a boiling step (95°C, 10 min). Nitrocellulose membranes are saturated 1 hour at RT with a blocking solution containing 5% (w/v) non-fat dry milk (BioRad) in PBS plus Tween-20 [PBS-T; in mM: 136.89 NaCl, 6.7 KCl, 20 Na_2HPO_4, 3.67 KH_2PO_4, 0.1% (w/v) Tween-20, pH 7.4], washed twice with PBS-T (10 min) and incubated 24 hours at 4°C with the primary antibody (6E10, Covance). The sample composition at 96 hours does not differ from that at 48 hours and it comprises monomers, dimers, trimers, tetramers and HMW oligomers, which are disrupted, alongside with the majority of tetramers and trimers, upon a 10-minute 95°C boiling procedure. See the enlarged monomer and dimer immuno-band in the boiled sample.

FIGURE 5. Quantitative reverse-transcription polymerase chain reaction (qPCR). Cortical neuronal cultures at DIV 11-13, seeded in 12-well culture vessels are washed twice with mKRB and then incubated for 1 h at 37°C with Aβ42o (0.5 µM) or vehicle (0.1% DMSO) [30]. Total RNA was extracted using the NucleoSpin RNA purification kit (Macherey-Nagel) and then quantified with NanoDrop 2000 (Thermo Scientific). For each sample, 500 ng of total RNA were reverse-transcribed into cDNA using the SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. PCR amplification was performed in an iQ5 Thermal Cycler (BioRad), using SYBR Green Supermix (BioRad) as reagent. For each cDNA, the efficiency of the reaction was estimated with a standard curve by using the cDNA reverse transcript from mouse brain. The transcript of Hypoxantine phosphoribosyl-transferase 1 (HPRT1) was used as internal control. The following primers (Invitrogen) were used:

RyR2: 5'-GGCTTGCTCCAGATGAAACT-3 and 5'-CTTCGATCCGGTGCCTAAC-3';

RyR3: 5'-CAAACTGCCCTCCCTAAACA-3' and 5'-AAACACGGTCCAAGAACAGC-3';

IP3R isoform 1: FW: 5'-CTCATGGACTGATTATGGACAGGAC-3'

and 5'-GCAGGTCAGCAAAGAACTTATAGCC-3';

IP3R- 3: 5'-ATAAGAAGGAGAGACCCTCGG-3' and 5'-CCACACATCTTGTTCAGCCT-3';

HPRT1: 5'- CTACAGAGTGCCTGACCT-3' and 5'-TCGTAGATCCCTCCGCATAC-3'.

Quantification followed the method of ΔCt comparison. Data are expressed as percentage ratio change relative to the HPRT1 transcript (mean ± SEM, n = number of wells).

FIGURE 1

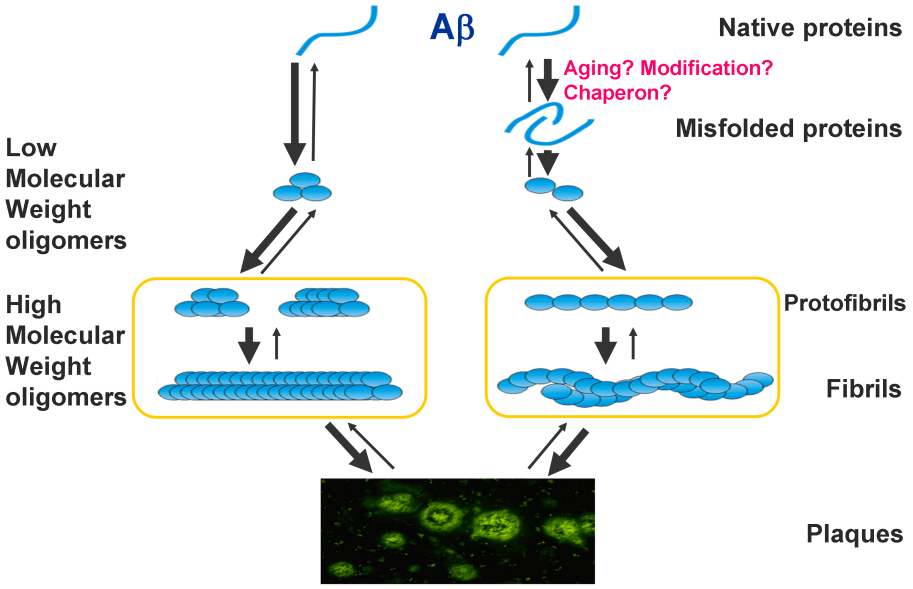


FIGURE 2

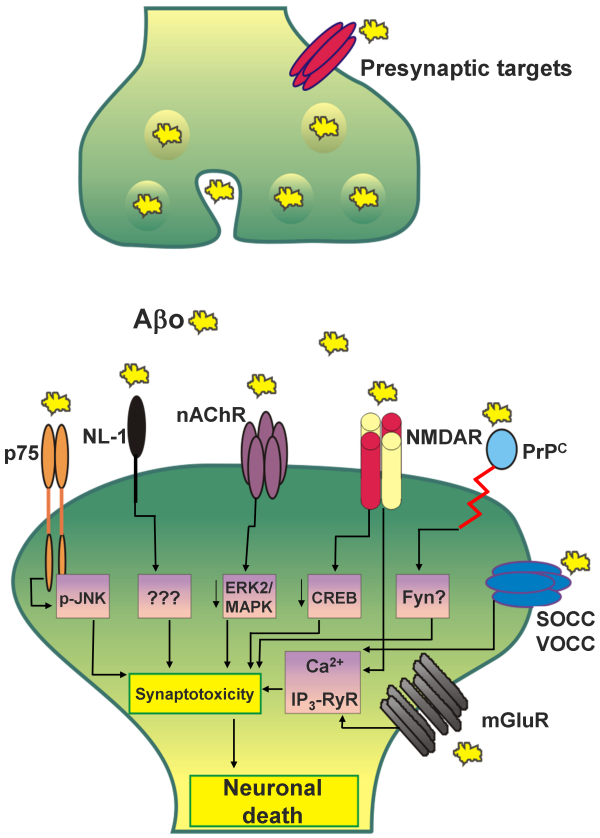


FIGURE 3

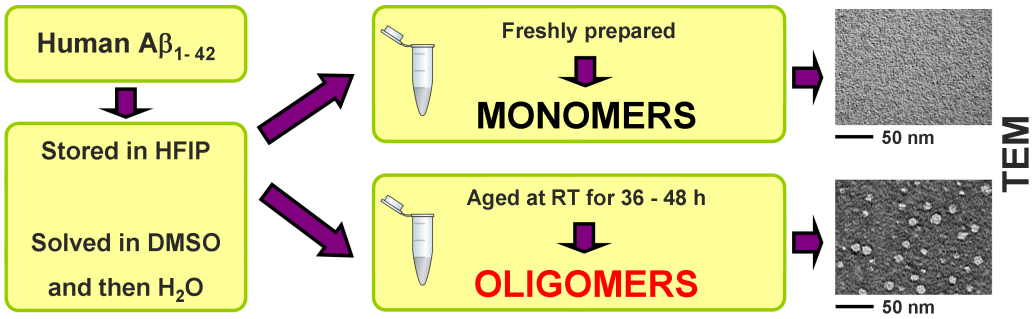


FIGURE 4

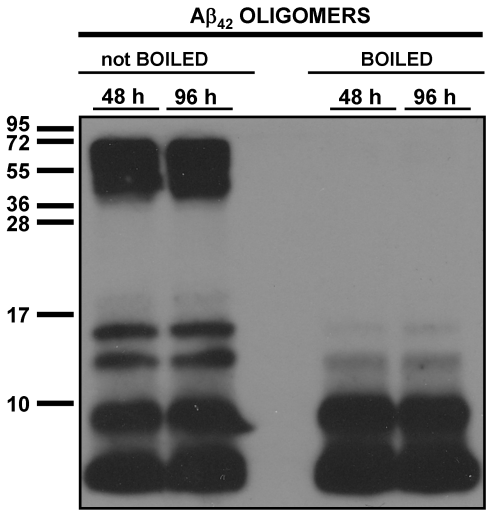
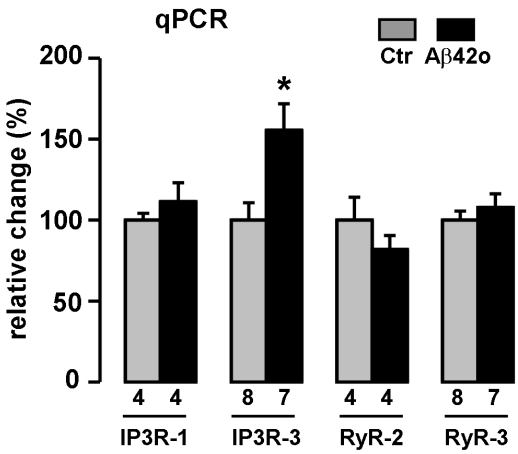


FIGURE 5



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